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## **2'-Modified Nucleosides for Site-Specific Labeling of Oligonucleotides**

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## 2'-Modified Nucleosides for Site-Specific Labeling of Oligonucleotides

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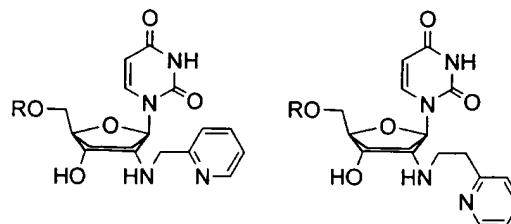
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We report the synthesis of 2'-modified nucleosides designed specifically for incorporating labels into oligonucleotides. Conversion of these nucleosides to phosphoramidite and solid support-bound derivatives proceeds in good yield. Large-scale synthesis of 11-mer oligonucleotides possessing the 2'-modified nucleosides is achieved using these derivatives. Thermal denaturation studies indicate that the presence of 2'-modified nucleosides in 11-mer duplexes has minimal destabilizing effects on the duplex structure when the nucleosides are placed at the duplex termini. The powerful combination of phosphoramidite and support-bound derivatives of 2'-modified nucleosides affords the large-scale preparation of an entirely new class of oligonucleotides. The ability to synthesize oligonucleotides containing label attachment sites at 3', intervening, and 5' locations of a duplex is a significant advance in the development of oligonucleotide conjugates.

### INTRODUCTION

Substituents at the 2'-ribose position of nucleosides are important structural and mechanistic probes of nuclease resistance and ribozyme catalysis (1). These nucleosides are used in the development of antisense therapeutics (1b) and the rapid screening of oligonucleotide sequences displaying high affinity toward protein targets (2). Nucleosides containing a primary amine at the 2' position facilitate the incorporation of several reporter molecules or labels into oligonucleotides (3). This is accomplished by (a) introducing the amine-containing nucleoside into an oligonucleotide using standard automated DNA synthesis, (b) purifying the resulting oligonucleotide, (c) reacting the reporter group with the amine-containing oligonucleotide, and (d) isolating the conjugate. Labels such as fluorescent dyes (3a,c), aromatic and aliphatic isocyanates (3b,d), and transition metal complexes (3e) have been successfully incorporated into oligonucleotides using this method.

Our objective is to develop new methods of incorporating labels, namely transition metal complexes, into DNA site-specifically (4). To this end we have designed nucleosides containing bidentate amine groups at the 2'-ribose position to which transition metal complexes are chelated (Figure 1). This site on the ribose ring is selected so that both solid support-bound and phosphoramidite forms of the nucleosides can be prepared. The solid support-bound nucleoside is used as the starting material in oligonucleotide synthesis, whereas the phosphoramidite monomer can be introduced at any later position in the oligonucleotide sequence. As a result, labels can be incorporated at the 3', intervening, or 5' locations of an oligonucleotide. We report the synthesis of 2'-modified nucleosides as both solid support-bound and phosphoramidite derivatives and their incorporation into oligonucleotides via solid-phase methods. Thermal denaturation studies of the resulting oligonucleotides indicate that the presence of the 2'-modified nucleosides has only a slight destabilizing effect on the duplex structure.



**Figure 1.** Structure of nucleosides with bidentate ligands at the 2' ribose position (R = 4,4'-dimethoxytrityl).

### EXPERIMENTAL PROCEDURES

**General.**  $^1\text{H}$  and  $^{31}\text{P}$  spectra were acquired on either Varian 300 or 500 spectrometers. Chemical shifts are reported in parts per million and referenced to the proton chemical shifts of deuterated solvent, trimethylsilane, or 85%  $\text{H}_3\text{PO}_4$  in  $\text{H}_2\text{O}$ . Reagents and starting materials were used as received from Aldrich. Flash chromatography was performed on EM Science/Merck silica gel 60 (230–400 mesh). Thin-layer chromatography (TLC) was performed on 0.25 mm Merck precoated silica plates (60 F<sub>254</sub>). Enzymes were purchased from Pharmacia. Mass spectrometry was performed at the Caltech Peptide/Protein Microanalytical Laboratory.

**5'-O-(4,4'-Dimethoxytrityl)-2,2'-O-anhydro-1-( $\beta$ -D-arabinofuranosyl)uracil, 1.** This compound was prepared from the reaction of 2,2'-O-anhydro-1-( $\beta$ -D-arabinofuranosyl)uracil and 4,4'-dimethoxytrityl chloride as previously described (5).

**5'-O-(4,4'-Dimethoxytrityl)-2'-N,3'-O-(2-oxooxazolidinyl)-2'-aminomethylpyridyl-2'-deoxyuridine, 2a.** To a solution of 1 (2.2 g, 4.2 mmol) in pyridine (40 mL) was added 1,1'-carbonyldiimidazole (1.01 g, 6.2 mmol). After 30 h of stirring at ambient temperature, the solvent was removed and the residue was resuspended in dichloromethane (40 mL); DIEA (1.1 mL, 6.3 mmol) and AMPy (649  $\mu\text{L}$ , 6.3 mmol) were delivered to the solution. After 60 h of stirring at ambient temperature, the reaction was quenched with 5% citric acid, extracted with fresh dichloromethane, dried over  $\text{Na}_2\text{SO}_4$ , and concentrated

to an oil. The residue was dissolved in tetrahydrofuran (25 mL), DBU (628  $\mu$ L, 4.2 mmol) was added to the flask, and the solution was refluxed for 46 h. The solvent was removed, and the residue was purified on silica (using 22% EtOAc in dichloromethane containing 1% TEA and 3% methanol) to afford **2a** in 20% yield (548 mg, 828  $\mu$ mol).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz)  $\delta$  3.48–3.58 (m, 2H), 3.78 (s, 6H), 4.35–4.40 (m, 1H), 4.54 (d, 1H), 4.75 (dd, 2H), 5.14–5.17 (m, 1H), 5.41 (d, 1H), 6.06 (d, 1H), 6.82 (d, 4H), 7.21–7.30 (m, 9H), 7.33–7.37 (m, 2H), 7.53 (d, 1H), 7.69 (t, 1H), 8.51 (d, 1H), 8.84 (s, 1H). ESI-MS mass calcd for  $\text{C}_{37}\text{H}_{35}\text{N}_4\text{O}_8$  ( $\text{M} + \text{H}^+$ ): 663.24. Found: 663.2.

**5'-O-(4,4'-Dimethoxytrityl)-2'-N,3'-O-(2-oxooxazolidinyl)-2'-aminoethylpyridyl-2'-deoxyuridine, 2b.** To a solution of **1** (1.0 g, 1.9 mmol) in pyridine (30 mL) was added 1,1'-carbonyldiimidazole (0.460 g, 2.8 mmol). After 30 h of stirring at ambient temperature, the solvent was removed by rotary evaporation and the residue was resuspended in dichloromethane (30 mL); DIEA (500  $\mu$ L, 2.85 mmol) and AEPy (340  $\mu$ L, 2.85 mmol) were delivered to the solution. After 60 h of stirring at ambient temperature, the reaction was worked up as described for **2a**. The residue was dissolved in THF (11 mL), DBU (284  $\mu$ L, 1.9 mmol) was added to the flask, and the solution was refluxed for 46 h. The solvent was removed by rotary evaporation, and the residue was purified on silica (eluting with 5–15% methanol in EtOAc) to afford **2b** in 10% yield (127 mg, 188  $\mu$ mol).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$  3.07–3.39 (m, 2H), 3.43–3.60 (m, 2H), 3.74–3.77 (m, 6H), 3.93–4.04 (m, 1H), 4.26–4.31 (m, 1H), 4.64 (dd, 2H), 5.09–5.14 (m, 1H), 5.35 (d, 1H), 6.09 (d, 1H), 6.84 (d, 4H), 7.15–7.39 (m, 11H), 7.59–7.69 (m, 2H), 8.57 (d, 1H). ESI-MS mass calcd for  $\text{C}_{38}\text{H}_{37}\text{N}_4\text{O}_8$  ( $\text{M} + \text{H}^+$ ): 677.25. Found: 677.2.

**5'-O-(4,4'-Dimethoxytrityl)-2'-aminomethylpyridyl-2'-deoxyuridine, 3a.** Compound **2a** (242 mg, 0.36 mmol) was suspended in dioxane (6 mL), 4 M NaOH (4.7 mL), and methanol (4.7 mL), and the mixture was stirred at 50  $^\circ\text{C}$  for 36 h. The solvents were removed by rotary evaporation, and the residue was dissolved in dichloromethane (55 mL). The solution was extracted with brine, dried over sodium sulfate, and evaporated to dryness to give **3a** in 92% yield (216 mg, 0.34 mmol).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$  3.3 (s(br), 4H), 3.73–3.78 (m, 6H), 3.94–4.02 (m, 1H), 4.1 (d, 1H), 4.2 (s, 1H), 5.3 (d, 1H), 6.1 (d, 1H), 6.7 (dd, 4H), 7.1–7.3 (m, 11H), 7.5–7.6 (m, 2H), 8.4 (s, 1H). ESI-MS mass calcd for  $\text{C}_{36}\text{H}_{37}\text{N}_4\text{O}_7$  ( $\text{M} + \text{H}^+$ ): 637.26. Found: 637.2.

**5'-O-(4,4'-Dimethoxytrityl)-2'-aminomethylpyridyl-2'-deoxyuridine, 3b.** Compound **2b** (1.7 g, 2.51 mmol) was suspended in dioxane (43 mL), 4 M NaOH (26 mL), and methanol (26 mL), and the mixture was stirred at room temperature for 20 h. The reaction was heated at 60  $^\circ\text{C}$  for an additional 2 h, after which the solvents were removed by rotary evaporation. The residue dissolved in dichloromethane, extracted with brine, dried over sodium sulfate, and evaporated to dryness. The material was purified on silica (eluting with 23% ethyl acetate in dichloromethane containing 1% each of methanol and triethylamine) to give **3b** in 88% yield (1.44 g, 2.21 mmol).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz)  $\delta$  3.03 (t, 2H), 3.12–3.25 (m, 2H), 3.43–3.53 (m, 3H), 3.80 (s, 6H), 4.29 (s, 1H), 4.49 (d, 1H), 5.37 (d, 1H), 5.94 (d, 1H), 6.14 (s(br), 1H), 6.85 (d, 4H), 7.18–7.33 (m, 10H), 7.40 (d, 1H), 7.67 (t, 1H), 7.74 (d, 1H), 8.49 (d, 1H). ESI-MS mass calcd for  $\text{C}_{37}\text{H}_{39}\text{N}_4\text{O}_7$  ( $\text{M} + \text{H}^+$ ): 651.27. Found: 651.2.

**5'-O-(4,4'-Dimethoxytrityl)-N<sup>2</sup>-trifluoroacetamido-2'-deoxyuridine, 4.** This compound was prepared either from reaction of 2'-amino-5'-O-(4,4'-dimethoxytrityl)-2'-

deoxyuridine and ethyl trifluoroacetate (**5**) or from reaction *N*<sup>2</sup>-trifluoroacetyl-2'-amino-2'-deoxyuridine and 4,4'-dimethoxytrityl chloride as previously described (**3a**).

**5'-O-(4,4'-Dimethoxytrityl)-2'-aminomethylpyridyl-2'-deoxyuridine-3'-O-succinate, 5.** Compound **3a** (100 mg, 0.157 mmol), DMAP (9.4 mg, 0.5 equiv), and succinic anhydride (17.3 mg, 1.1 equiv) were suspended in dry pyridine (1 mL) and allowed to stir under argon for 2 h, at which time an additional 0.2 equiv of succinic anhydride was added. The reaction proceeded overnight at room temperature, after which the solvent was removed. The residue was dissolved in dichloromethane (20 mL) and stirred with an equal volume of 5%  $\text{NaHCO}_3$  solution for 2 h. The mixture was partitioned, and the organic phase was extracted with brine. Each phase was back-extracted once. The combined organic phases were washed with cold 5% citric acid and concentrated to a small volume (3–5 mL). The sample was precipitated in stirring hexanes, and the white solid **5** was collected by filtration in 60% yield (69 mg, 0.094 mmol).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CD}_3\text{CN}$ )  $\delta$  178.82, 174.10, 165.67, 160.11, 158.27, 156.03, 146.0, 145.95, 136.96, 136.76, 136.6, 133.06, 131.32, 129.47, 128.77, 127.74, 122.9, 114.45, 114.0, 102.0, 88.12, 85.33, 73.0, 72.88, 65.04, 56.03, 30.98. ESI-MS mass calcd for  $\text{C}_{40}\text{H}_{39}\text{N}_4\text{O}_{10}$  ( $\text{M} - \text{H}^-$ ): 735.27. Found: 735.2.

**5'-O-(4,4'-Dimethoxytrityl)-N<sup>2</sup>-trifluoroacetamido-2'-deoxyuridine-3'-O-succinate, 6.** Compound **4** (100 mg, 0.156 mmol), DMAP (9.4 mg, 0.5 equiv), and succinic anhydride (17.2 mg, 1.1 equiv) were suspended in dry pyridine (2 mL) and stirred under argon for 2 h. An additional 0.2–0.5 equiv of succinic anhydride was added, and the reaction proceeded at room-temperature overnight. The reaction was worked according to the procedure given for **5**. A white powder **7** was isolated in 74% yield (85 mg, 0.115 mmol).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CD}_3\text{CN}$ )  $\delta$  175.04, 172.80, 163.85, 160.13, 140.71, 131.42, 130.35, 129.32, 128.96, 128.43, 114.60, 114.23, 103.97, 86.30, 84.29, 74.18, 64.77, 56.42, 55.77, 30.49. ESI-MS mass calcd for  $\text{C}_{36}\text{H}_{33}\text{N}_3\text{O}_{11}$  ( $\text{M} - \text{H}^-$ ): 740.21. Found: 740.2.

**5'-O-(4,4'-Dimethoxytrityl)-2'-aminomethylpyridyl-2'-deoxyuridine-3'-O-succinated Support, 7.** Compound **7** was prepared by suspending the solid support (controlled pore glass derivatized with long-chain alkylamine, 500 Å pore size, 350 mg) in dry dichloromethane (5 mL) and adding TEA (250  $\mu$ L) and **5** (180 mg, 245  $\mu$ mol); HOBT (33 mg, 245  $\mu$ mol) and BOP (119 mg, 270  $\mu$ mol) were added to the suspension. The mixture was agitated for 16 h at room temperature, filtered, and washed with dichloromethane (2  $\times$  10 mL). The solid was transferred to a separate flask and suspended in pyridine (7.5 mL). Acetic anhydride (1–2 mL) and *N*-methylimidazole (100  $\mu$ L) were added to the flask, and the mixture was agitated overnight. The solid was filtered and washed with pyridine (3  $\times$  10 mL), methanol (3  $\times$  10 mL), dichloromethane (3  $\times$  10 mL), and diethyl ether (3  $\times$  10 mL). The white solid **7** was dried in vacuo. The nucleoside loading was determined spectrophotometrically to be 60  $\mu\text{mol/g}$  (**6**).

**5'-O-(4,4'-Dimethoxytrityl)-N<sup>2</sup>-trifluoroacetamido-2'-deoxyuridine-3'-O-succinated Support, 8.** Compound **8** was prepared using the same method described for **7**. The nucleoside loading for **8** was 52  $\mu\text{mol/g}$ .

**5'-O-(4,4'-Dimethoxytrityl)-2'-aminomethylpyridyl-2'-deoxyuridine-3'-O-(2-cyanoethyl *N,N*-diisopropylaminophosphoramidite), 9.** While under argon **3a** (200 mg, 0.314 mmol) was dissolved in dry dichloromethane (2 mL) containing DIEA (220  $\mu$ L, 4 equiv). The reaction vessel was degassed several times prior to

the addition of 2-chlorocyanoethyl *N,N*-diisopropylaminophosphoramidite (105  $\mu$ L, 1.5 equiv) dropwise over 5 min. A positive ninhydrin test indicated formation of the desired product. After 50 min the reaction was diluted with 200  $\mu$ L of methanol and evaporated to dryness. The residue was applied to silica (eluting with 0–80% dichloromethane in hexane containing 1% TEA) to give an off-white powder **9** in 57% yield (151 mg, 0.180 mmol). ESI-MS mass calcd for  $C_{45}H_{52}N_6O_8P$  ( $M - H$ )<sup>−</sup>: 835.37. Found: 835.4.

**5'-O-(4,4'-Dimethoxytrityl)-N<sup>2</sup>-trifluoroacetamido-2'-deoxyuridine-3'-O-(2-cyanoethyl *N,N*-diisopropylaminophosphoramidite), **10**.** While under argon **4** (100 mg, 0.163 mmol) was dissolved in dry dichloromethane (2 mL) containing DIEA (110  $\mu$ L, 4 equiv). The reaction vessel was degassed several times prior to the addition of 2-chlorocyanoethyl *N,N*-diisopropylaminophosphoramidite (53  $\mu$ L, 1.5 equiv) dropwise over 5 min. A positive ninhydrin test indicated formation of the desired product. After 90 min the reaction was diluted with ethyl acetate which had been previously washed with cold 10% sodium carbonate. The organic layer was extracted twice with cold 10% sodium carbonate and once with brine. The organic fraction was dried over sodium sulfate and evaporated to an oil. The residue was purified on silica (eluting with 20% hexane in dichloromethane containing 2% methanol and 1% TEA) to yield an off-white powder **10** in 77% yield (105 mg, 0.125 mmol). <sup>1</sup>H NMR ( $CD_3CN$ , 300 MHz)  $\delta$  1.26 (d, 12H), 3.4 (m, 2H), 3.5 (m, 2H), 3.6–3.8 (m, 3H), 3.79 (s, 6H), 4.1 (m, 2H), 4.3 (m, 1H), 4.4 (m, 1H), 4.5 (m, 1H), 4.7 (m, 1H), 4.8 (m, 1H), 5.4 (d, 1H), 5.8 (s, 1H), 6.1 (d, 1H), 6.9 (m, 4H), 7.3 (m, 9H), 7.5 (m, 2H), 7.6 (t, 1H), 7.9 (s, 1H). <sup>31</sup>P NMR ( $CD_3CN$ , 300 MHz)  $\delta$  153.13 and 154.06 ppm observed for the diastereomers. ESI-MS mass calcd for  $C_{41}H_{46}F_3N_5O_9P$  ( $M - H$ )<sup>−</sup>: 840.31. Found: 840.2.

**Oligonucleotide Synthesis.** Protected deoxyribonucleoside phosphoramidites and other reagents required for solid-phase DNA synthesis were purchased from Applied Biosystems, Incorporated (ABI). All oligonucleotides were synthesized on a 1.0  $\mu$ mole scale. Solid supports (**7**, **8**) were packed in column assemblies purchased from ABI and contained approximately 23 mg of derivatized resin, depending on the nucleoside loading. The initial coupling steps in each synthesis were increased from 30 s to 2 min. The concentration of phosphoramidites (**9**, **10**) typically ranged from 0.1 to 0.18 M in dry acetonitrile. The coupling time for **9** and **10** was 15 min.

Oligonucleotides **11**, **13**, and **14** were synthesized with the terminal dimethoxytrityl (DMT) group retained. Oligonucleotide **12** was prepared with the 5'-DMT group removed prior to cleavage and deprotection. Oligonucleotides **11**–**13** were cleaved from the solid support with concentrated ammonia during the automated synthesis routine and deprotected for either 16 h at 55 °C or 4 h at 65 °C. Oligonucleotide **14** was cleaved manually in 5 mL of concentrated ammonia for 16 h at 55 °C. Oligonucleotides **15**–**18** were prepared by standard trityl-off procedures.

**Oligonucleotide Purification.** Deprotected oligonucleotides containing a 5'-DMT group were suspended in 20% triethylammonium bicarbonate buffer (1.0 M, pH 8.5) in water and injected onto a reverse phase VYDAC 201HS1022 C18 column. Preparative HPLC was performed with a Waters 600E Controller and 994 Diode Array Detector, using the following gradient: 0–100% B over 50 min, where A = 0.1 M triethylamine acetate, 2% acetonitrile; B = 0.05 M triethylamine acetate, 50%

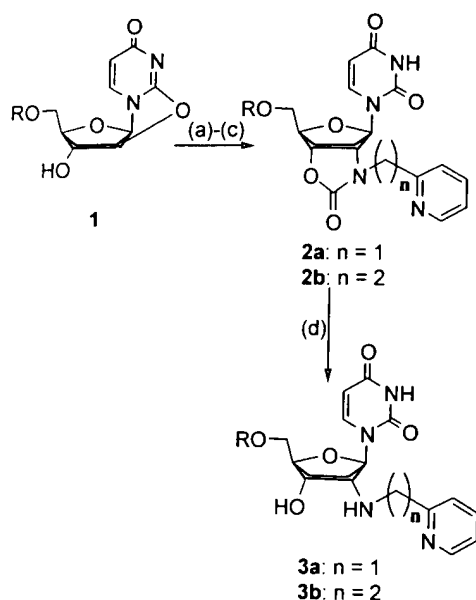
acetonitrile. The collected peaks were dried in vacuo and further purified using Waters C18 Classic SepPak cartridges. The amount of purified oligonucleotide was determined spectrophotometrically, with  $\epsilon_{260}$  values given in parentheses: **11** (119800 M<sup>−1</sup> cm<sup>−1</sup>), **12** (115150 M<sup>−1</sup> cm<sup>−1</sup>), **13** (95900 M<sup>−1</sup> cm<sup>−1</sup>), **14** (97250 M<sup>−1</sup> cm<sup>−1</sup>).

Oligonucleotides **11**, **13**, and **14** were detritylated according to the procedure outlined by ABI manual and desalted using SepPak cartridges (**7**). The detritylated oligonucleotides were further purified using a weak anion exchange column purchased from SynChroPak (AX-100, analytical, semiprep), using the following gradient: 0–90% B over 35 min, where A = 50 mM sodium phosphate (pH 5.9), 30% methanol; B = 50 mM sodium phosphate (pH 5.9), 30% methanol, 1 M ammonium sulfate. The collected peaks were desalted using SepPak cartridges and assayed as described above.

**Oligonucleotide Yield.** The overall yield of each modified oligonucleotide, following HPLC purification and workup, ranged from 30 to 40%, based on a 12- $\mu$ mol synthesis. The detritylated, purified oligonucleotides **11**–**14** were characterized by MALDI-TOF mass spectrometry: **11**, calculated, 3355 [M], found, 3356.24 [M – H]<sup>−</sup>; **12**, calculated, 3408 [M], found, 3407.17 [M – H]<sup>−</sup>; **13**, calculated, 3230 [M], found, 3228.38 [M – H]<sup>−</sup>; **14**, calculated, 3320 [M], found, 3319.43 [M – H]<sup>−</sup>.

**Enzymatic Digestion of Oligonucleotides and HPLC Analysis.** Approximately 10–30 nmol of purified oligonucleotide was subjected to enzymatic digestion analysis. The digest cocktail (55  $\mu$ L/sample) contained bacterial alkaline phosphatase (4  $\mu$ L, 10  $\mu$ L/unit) and snake venom phosphodiesterase (2.4  $\mu$ L, 1 mL/mg), in 1 M MgCl<sub>2</sub> (0.8  $\mu$ L), 0.5 M Tris buffer, pH 7.5 (3.5  $\mu$ L). The reaction mixture was incubated at 37 °C for 8–16 h. The reaction was stopped by adding 3 M sodium acetate (7  $\mu$ L) and ethanol (155  $\mu$ L) to the samples, which were then frozen on dry ice (10 min) and centrifuged (10 min) at 4 °C. The supernatants were removed and transferred to new tubes, each containing 452  $\mu$ L of ethanol. The samples were frozen and centrifuged; the resulting supernatants were removed and dried in vacuo. The samples were dissolved in water (200  $\mu$ L) and injected onto a reverse phase Vydac (201HS54 4.6 mm  $\times$  25 cm, 5  $\mu$ m, 90 Å) or Prism (Keystone Scientific, 4.6  $\times$  250 mm, 5  $\mu$ m, 100 Å) C18 column. The product nucleosides were eluted within 20 min, according to either of the following gradients: (Vydac) 0–30% B over 20 min then 30–100% B over 10 min, where A = 0.1 M triethylamine acetate, 2% acetonitrile, B = 0.05 M triethylamine acetate, 50% acetonitrile; (Prism) 0–17% B over 15 min then 17–75% B over 18 min, where A = 0.1 M triethylamine acetate, pH 7.0, 2% acetonitrile, B = 100% acetonitrile. The resulting peaks were compared against the appropriate set of nucleoside standards for a given oligonucleotide sequence.

**Thermal Denaturation Measurements.** Individual oligonucleotides were hybridized to their complementary strands in 50 mM NaP<sub>i</sub> buffer (pH 7.0), 0.5 M NaCl, to give solutions that were 2.7  $\mu$ M in each strand. The samples were heated for 20 min at 70 °C and cooled to 4 °C overnight. Thermal denaturation profiles were measured at 260 nm with an Hewlett-Packard diode array UV-vis spectrophotometer equipped with a Peltier temperature controller and interfaced with a personal computer. Samples were equilibrated at 20 °C for 10–20 min prior to data collection. Absorbance values were taken over a temperature range of 20–70 °C, with measurements made every 0.5 °C with an equilibration time of 60 s for each point. Each hybrid went through 2–4

Scheme 1<sup>a</sup>

<sup>a</sup> Reagents: (a) (imid)<sub>2</sub>CO, pyridine, rt, 30 h; (b) NH<sub>2</sub>R (R = CH<sub>2</sub>pyr, CH<sub>2</sub>CH<sub>2</sub>pyr), DIEA, CH<sub>2</sub>Cl<sub>2</sub>, rt, 60 h; (c) DBU, THF, reflux, 46 h; (d) 6 N NaOH, dioxane, CH<sub>3</sub>OH, 50 °C, 36 h. Abbreviation: R = 4,4'-dimethoxytrityl.

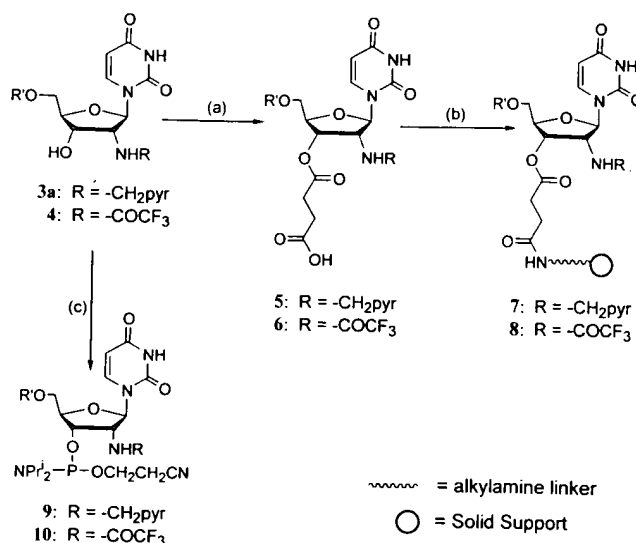
separate heat-cool cycles, and the  $T_m$  values obtained from these heating and cooling traces were averaged to give the final  $T_m$  value. Standard deviations were calculated for each duplex.

## RESULTS

**Synthesis of 2'-Modified Nucleosides.** Nucleosides with bidentate amine groups such as aminomethylpyridine (AMPy) (8) and aminoethylpyridine (AEPy) at the 2' ribose position were prepared as shown in Scheme 1. This approach was based on methods developed recently for incorporating 2'-N-alkylamino substituents into nucleosides (9). Nucleoside 1 was converted to the 3'-N-alkyl carbamate upon prolonged treatment with carbonyl diimidazole in pyridine; this was followed by the addition of AMPy or AEPy in the presence of DIEA and dichloromethane. Subsequent cyclization in THF using the cyclization agent DBU produced either 2a or 2b in yields of 20% and 10%, respectively. Prolonged heating of 2a and 2b in a basic dioxane-methanol solution caused deprotection at the 2',3'-positions, giving 3a and 3b in high yield (92% and 88%, respectively).

Derivatization of solid supports with 2'-modified nucleosides was achieved according to Scheme 2. Nucleoside 4 was prepared according to previously published procedures (3a, 5, 10). Both 3a and 4 were treated with succinic anhydride to give the corresponding hemisuccinates 5 and 6 in yields of 60% and 74%, respectively (6, 11). Solid supports such as controlled pore glass (CPG) containing long-chain alkylamine groups were derivatized with 6 using *p*-nitrophenol and DCC. This method resulted in solid supports with low nucleoside loading. Subsequent attempts to prepare the nucleoside-modified solid supports employed the coupling agent BOP in the presence of HOBT and TEA (12). The unreacted amine groups were treated with acetic anhydride, and the nucleoside loadings of the solid supports 7 and 8 were determined by spectrophotometric assay (60 and 52 μmol/g, respectively) (6).

The preparation of phosphoramidite derivatives of nucleosides 3a and 4 relied on standard methods (3a, 6,

Scheme 2<sup>a</sup>

<sup>a</sup> Reagents: (a) succinic anhydride, pyridine, DMAP, rt, 16 h; (b) solid support, TEA, HOBT, BOP, CH<sub>2</sub>Cl<sub>2</sub>, rt, 16 h; acetic anhydride, *N*-methylimidazole, pyridine, rt, 12 h; (c) amidite, DIEA, CH<sub>2</sub>Cl<sub>2</sub>, rt, 50 min. Abbreviation: R' = 4,4'-dimethoxytrityl.

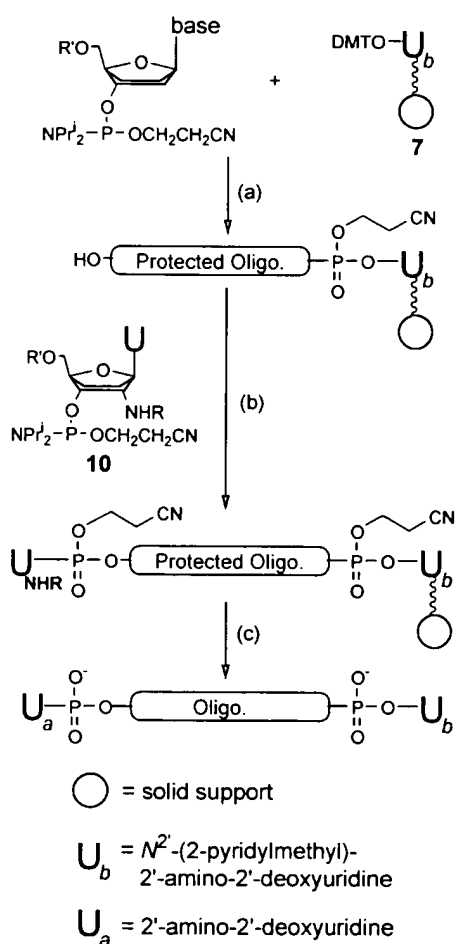
10). Nucleosides 3a and 4 were treated with 2-chloro-cyanoethyl *N,N*-diisopropylaminophosphoramidite in the presence of DIEA to give 9 and 10 in 57% and 77% yield, respectively (Scheme 2).

**Oligonucleotide Synthesis.** A series of oligonucleotides were prepared from the support-bound and phosphoramidite nucleoside derivatives as outlined in Scheme 3 (see Table 1 for sequences). To maximize coupling, the reaction time for the first step was increased from 30 s to 2 min (yield > 95%). The reaction times for 9 and 10 were 15 min in length, leading to coupling yields > 90%.

All oligonucleotides were cleaved from the solid support with concentrated ammonia as a part of the automated synthesis routine, except in the case of 14, which was manually cleaved. The yield of purified oligonucleotide for 11–14 ranged from 30 to 40%, which was comparable to those values observed for 15–18. Results from MALDI-TOF mass spectrometry on 11–14 were in excellent agreement with the calculated values. Further characterization of these oligonucleotides was achieved by enzymatic digestion with alkaline phosphatase and phosphodiesterase (Figure 2) (7). Analysis of the digestion products showed the expected distribution of nucleosides determined for each oligonucleotide sequence (13).

**Thermal Denaturation Studies.** We investigated the thermal stability of duplexes containing 2'-modified nucleosides. Table 2 shows the transition melting temperatures ( $T_m$ ) for each duplex prepared in 50 mM sodium phosphate buffer (pH 7.0) containing 0.5 M sodium chloride. The  $T_m$  of the 11-mer duplex formed by the unmodified oligonucleotides 15 and 16 was 48 °C. When one 5'-terminal nucleoside from this duplex was substituted with either 2'-amino-2'-deoxyuridine (U<sub>a</sub>) or *N*<sup>2</sup>-(2-pyridylmethyl)-2'-amino-2'-deoxyuridine (U<sub>b</sub>), the  $T_m$  value remained unchanged ( $T_m$  = 47 °C for duplexes 11: 15 and 12:16). When both U<sub>a</sub> and U<sub>b</sub> were incorporated at the 5' ends of the same duplex (11:12), the  $T_m$  value decreased to 45 °C.

The small change in the melting profile was similar to the results obtained with duplexes of identical length, GC content, and type of nucleoside modification, but of different sequence. This second set of duplexes contained

Scheme 3<sup>a</sup>


<sup>a</sup> Steps in the synthesis of oligonucleotide 14: (a) detritylation of 7; monomer coupling, normal synthesis cycle; (b) coupling of 10; (c) cleavage and deprotection. R = COCF<sub>3</sub>, R' = 4,4'-dimethoxytrityl.

Table 1. Oligonucleotide Sequences<sup>a</sup>

sequence	abbreviation
5'- $U_a$ CAGCTGTAGA	11
5'- $U_b$ CTACAGCTGA	12
5'- $U_a$ CTCCTACACU <sub>a</sub>	13
5'- $U_a$ CTCCTACACU <sub>b</sub>	14
5'-TCTACAGCTGA	15
5'-TCAGCTGTAGA	16
5'-TCTCCTACACT	17
5'-AGTGTAGGAGA	18

<sup>a</sup> The symbol  $U_a$  denotes 2'-amino-2'-deoxyuridine;  $U_b$  denotes  $N^{2'}$ -(2-pyridylmethyl)-2'-amino-2'-deoxyuridine.

2'-modified nucleosides at both the 3' and 5' ends of the same strand. Duplex 13:18 contained  $U_a$  nucleosides at the 5' and 3' termini, whereas duplex 14:18 contained  $U_a$  at the 5' end and  $U_b$  at the 3' end. The  $T_m$  values (46 °C) for duplexes 13:18 and 14:18 decreased slightly in comparison to the value of the unmodified duplex 17:18 ( $T_m$  = 48 °C; Figure 3).

## DISCUSSION

**Synthetic Strategy.** Two general methods exist for preparing oligonucleotides containing transition metal complexes. One method involves the synthesis of nucleosides that possess metal-binding ligands, followed by incorporation of these modified nucleosides into oligonucleotides and subsequent metal complexation at the specific attachment sites (3e, 14). The second method

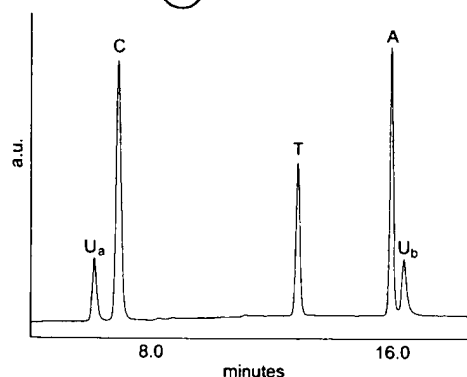


Figure 2. Products of enzymatic digestion of 14 as analyzed by reverse-phase HPLC.

Table 2. Thermal Denaturation Temperatures for Oligonucleotides Containing 2'-Substituted Nucleosides

duplex	$T_m$ (°C)	modification <sup>b</sup>
15:16	48.2 ± 0.5	none
11:15	46.6 ± 0.4	5' $U_a$
12:16	46.7 ± 0.4	5' $U_b$
11:12	45.0 ± 0.5	5' $U_a$ , 5' $U_b$
17:18	47.6 ± 0.2	none
13:18	45.8 ± 0.5	5' $U_a$ , 3' $U_a$
14:18	46.2 ± 0.5	5' $U_a$ , 3' $U_b$

<sup>a</sup> Values determined in 50 mM NaPi buffer (pH 7.0) containing 0.5 M NaCl. The concentration of each oligomer was 2.7 μM. <sup>b</sup> The symbol  $U_a$  denotes 2'-amino-2'-deoxyuridine;  $U_b$  denotes  $N^{2'}$ -(2-pyridylmethyl)-2'-amino-2'-deoxyuridine.

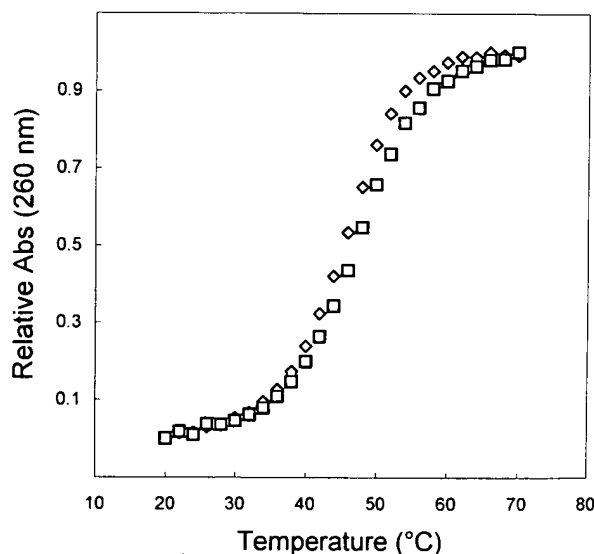


Figure 3. Thermal denaturation curves for the unmodified duplex 17:18 (□) and modified duplex 14:18 (◇).

entails the synthesis of metal-containing nucleosides that are incorporated during solid-phase oligonucleotide synthesis (4ab, 15). The 2'-modified nucleosides described in this work can be used in either method, resulting in oligonucleotides site-specifically labeled with metal complexes (3e, 4).

The choice of 2'-substituents described here are based on ligands that avidly bind transition metal complexes, such as AMPy and AEPy (16). The site-specific labeling of oligonucleotides with metal reagents is of considerable interest for several reasons. Recent experiments involving ruthenium-modified duplexes have shown that DNA can mediate energy and electron transfer reactions (3e, 15f, 17). Additionally, metal-modified primers have been used in dideoxy DNA sequencing techniques and elec-

trochemistry-based DNA assays (18). Therefore, nucleosides containing metal-binding ligands such as those in Figure 1 can facilitate the use of metal complexes as probes of nucleic acid structure and function (3e, 4).

**Synthesis of 2'-Modified Nucleosides.** The synthesis of nucleosides containing 2'-*N*-alkylamino substituents is an extension of methods developed by Sebesta, McGee, and co-workers (9). The yields of isolation determined for **2a** and **2b** are lower than those observed for nucleosides containing similarly bulky substituents and may be attributed to the purification conditions required for DMT-protected vs silyl-protected intermediates (9a). The products **3a** and **3b** are isolated in yields comparable to the yields reported by Sebesta for similar 2'-modified nucleosides.

The preparation of solid supports derivatized with 2'-modified nucleosides is complicated by the poor steric accessibility of the 3' ribose site (19). The steric bulk at the 2' position hinders the reaction of the 3' hydroxyl with succinic anhydride. Succination of **3a** and **4** proceeds in reasonable yields and demonstrates that both small and large functional groups at the 2' position can be tolerated in the succination step. High nucleoside loadings for **7** and **8** are achieved using the coupling agent BOP and an excess of nucleoside hemisuccinate in the derivatization step. While the solid support employed here is glass-based, the method is applicable to other supports containing long-chain alkylamine linkers.

Successful derivatization of supports with nucleosides such as **3a** and **4** affords the synthesis of 3' oligonucleotide conjugates in which the label is incorporated directly on the ribose ring. This is a significant advance since the current preparation of 3' oligonucleotide conjugates is achieved with supports containing either nucleosides with base-tethered primary amine groups (20) or nonnucleosidic amine derivatives (21). The ribose is an attractive attachment site since the labels introduced here cause fewer perturbations to the secondary duplex structure than labels attached to the nucleoside base (4). Additionally, the absence of a long linker between the attachment site and the incorporated label minimizes disruptions to the hydrogen bonding capacity of the oligonucleotide conjugate.

**Oligonucleotide Synthesis with 2'-Modified Nucleosides.** The successful large-scale synthesis of several 11-mer oligonucleotides validates the utility of 2'-modified nucleosides as support-bound and phosphoramidite derivatives (Table 1). Oligonucleotide synthesis beginning with **7** or **8** proceeds with minor modification to the automated protocol. The coupling yields of phosphoramidites **9** and **10** are acceptable for routine oligonucleotide preparation, and the purification of the several 11-mer modified oligonucleotides is straightforward. The isolation yields for **11**–**14** are comparable to values determined for oligonucleotides **15**–**18** under identical synthetic and purification conditions, implying that the use of 2'-modified nucleosides does not compromise the overall yield.

**Effect of 2'-Modified Nucleosides on Duplex Stability.** The presence of nucleosides containing ribose substituents at the 2' position causes slight destabilization to the modified duplexes, as assessed by thermal denaturation studies. Interestingly, analysis of the transition melting ( $T_m$ ) temperatures listed in Table 2 suggests that the  $T_m$  values are influenced by the number of 2'-modified nucleosides present in a duplex, not the size of the 2' substituent. For example, thermal denaturation of duplexes **11:15** and **12:16** produces identical  $T_m$  values, despite the difference in the size of the 2'

substituent ( $-NMe_2$  vs AMPy). The  $T_m$  of **11:12** shows the effect of placing two 2'-modified nucleosides at the 5' ends of the duplex. Similar results are obtained with a second set of duplexes identical in length, GC content, and type of nucleoside modification. The placement of two 2'-modified nucleosides at the 5' and 3' ends of the same strand results in duplexes destabilized by 1–2 °C (**13:18** and **14:18** vs **17:18**). We conclude that the extent of duplex destabilization is the same when two 2'-modified nucleosides are introduced at either (1) the 5' and 3' ends of a one strand hybridized to its complement, or (2) the 5' ends of complementary strands.

Compared to 2'-deoxynucleosides, 2'-aminonucleosides favor the 2'-endo sugar conformation to a higher degree (22). This observation suggests that 2'-aminonucleosides should stabilize DNA/DNA duplexes. However,  $T_m$  data for a series of 9-mer duplexes containing a 2'-aminonucleoside in the middle of the sequence indicate that the presence of these modified nucleosides has a destabilizing effect (**3a**). Our work shows that placement of the 2'-amino-modified nucleosides at the ends of the duplexes minimizes the destabilization imposed by the altered sugar conformation.

## CONCLUSION

We report the synthesis of 2'-modified nucleosides designed specifically for incorporating reporter molecules into oligonucleotides. Because these nucleosides contain modifications to the ribose ring (as opposed to the nucleobase), they are important contributions to current library of modified nucleosides. The introduction of metal-binding ligands at the 2' position is achieved after two steps. Conversion of these nucleosides to solid support-bound and phosphoramidite derivatives proceeds in good yield. The powerful combination of these derivatives affords the preparation of an entirely new class of oligonucleotide conjugates—those which contain label attachment sites at 3', intervening, and 5' sites of a duplex.

Thermal denaturation studies indicate that the presence of 2'-modified nucleosides in 11-mer duplexes has a slight destabilizing effect on the duplex structure. This effect is limited by the selective placement of these nucleosides at the ends of the duplexes. Interestingly, the size of the metal-binding substituent does not influence the magnitude of the destabilization. This characteristic makes these 2'-modified nucleosides even more attractive for use in the site-specific incorporation of reporter molecules into oligonucleotides.

The methodologies employed here can be extended to other modified nucleosides. Succination yields for nucleosides containing metal-binding ligands at locations other than the 2' position are expected to be much higher due to the absence of steric constraints. While the solid support is glass-based, the method is applicable to other solid supports containing any long-chain alkylamine linker. The library of solid supports containing modified nucleosides can be significantly expanded with the coupling conditions described here. These nucleoside reagents will enable the incorporation of labels known to be useful probes of nucleic acid structure and function.

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**Supporting Information Available:** MALDI-TOF mass spectra for 11–13. HPLC of digestion products of 11–13. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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